Potent Thrombin Inhibitors That Probe the S_1' Subsite: Tripeptide Transition State Analogues Based on a Heterocycle-Activated Carbonyl Group[1]

Michael J. Costanzo,[†] Bruce E. Maryanoff,*[,†] Leonard R. Hecker,[†] Mary R. Schott,[†] Stephen C. Yabut,[†] Han-Cheng Zhang,[†] Patricia Andrade-Gordon,[†] Jack A. Kauffman,[†] Joan M. Lewis,[†] Raman Krishnan,[‡] and Alexander Tulinsky[‡]

Drug Discovery, The R. W. Johnson Pharmaceutical Research Institute, Spring House, Pennsylvania 19477, and Department of Chemistry, Michigan State University, East Lansing, Michigan 48824

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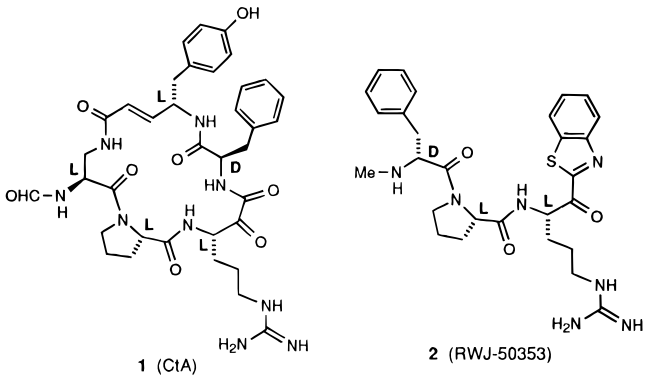
The pathology of thrombosis entails inappropriate activity of the hemostatic mechanism, which results in the interruption of normal blood flow and associated damage to cells, tissues, and organs. Because this condition is a serious source of mortality and morbidity in patients worldwide, new types of antithrombotic therapeutic agents are needed. Inhibitors of the serine protease α-thrombin (EC 3.4.21.5), an enzyme that is central to the regulation of hemostasis and thrombosis, could represent potentially useful drugs in this area.[2−9]

In the area of thrombin inhibitors, we have been investigating transition state analogues[10] with a cyclic structure, such as cyclotheonamide A (**1**) and its congeners,[11] and with an acyclic structure based on the classical tripeptide motif D-Phe-Pro-Arg.[2,5−7] Besides having the appropriate elements for molecular recognition in the active site of thrombin, particularly for the S_1 specificity pocket and the apolar S_2 and S_3 subsites, these molecules also have an electrophilic arginine carbonyl that can form a hemiacetal adduct with Ser-195 of the catalytic triad as part of a complex hydrogen-bonding network. At the outset of our work in 1991, we sought to identify tripeptide transition state analogues of the general form D-Phe-Pro-Arg-X, with new types of carbonyl-activating groups, "X". It occurred to us that the use of certain heterocyclic entities for "X", such as 2-oxazolyl or 2-pyridyl ("Het"), would likely afford the necessary electrophilicity to the arginine carbonyl and thus provide an effective solution. We favored this previously untested approach to enzyme inhibitors because (1) the use of different, suitable heterocycles, or substitution of a suitable heterocycle, would modulate carbonyl electrophilicity and (2) the heterocycle would provide a template for probing novel molecular recognition within the underutilized S_1' domain of the thrombin active site.

While we were pursuing this project, Edwards et al. published a seminal paper in 1992 describing the first examples of heterocycle activation in the design of novel inhibitors of the serine protease elastase.[12] In fact, an X-ray crystal structure of Ac-Val-Pro-Val-(2-benzoxazole) complexed with porcine pancreatic elastase (PPE) suggested a new hydrogen-bonding interaction between the benzoxazole nitrogen and His-57 of the catalytic triad.[12] More recently, Edwards et al.[13] published two additional papers on analogous elastase inhibitors, which appear quite effective, and Tsutsumi et al.[14] described α-keto heterocyclic inhibitors of prolyl endopeptidase. We now wish to report on the synthesis and biological activity of peptidoyl heterocycles with the structural motif Me-(D-Phe)-Pro-Arg-Het, some of which are potent inhibitors of thrombin and trypsin (Table 1). Moreover, via X-ray crystallography, we determined the molecular structure of a complex between the subnanomolar inhibitor **2** and human α-thrombin, which depicts some key interactions in the S_1' region of the active site: The benzothiazole ring forms a hydrogen bond with His-57 and an aromatic stacking interaction with Trp-60D of thrombin's unique insertion loop.[15]



1 (CtA) **2** (RWJ-50353)

Synthetic Chemistry. The tripeptidoyl heterocycles (Table 1) were generally synthesized by two routes, which are illustrated for the synthesis of **2** in Schemes 1 and 2.[17] In the first route, the heterocycle was formed from keto imidate **A** (prepared by reacting aldehyde **B**[19] with acetone cyanohydrin[12] and treating the adduct with methanolic HCl) and an appropriate bifunctional amine (Scheme 1). Reaction of **A** with 2-aminothiophenol furnished benzothiazole **C**, which was oxidized with the Dess−Martin reagent,[20] deprotected with HF, and purified to furnish **2**.

In the second route, the heterocycle was introduced by reacting Weinreb amide **D**[21] with an appropriate lithio heterocycle at low temperature (Scheme 2). Excess lithium reagent is required to overcome quenching caused by the exchangeable protons. Arylsulfonyl protecting groups are preferred for the Arg guanidine since Cbz and Fmoc groups are cleaved under these reaction conditions. Hence, treatment of **D** with 5−8 mol equiv of 2-lithiobenzothiazole at −78 °C furnished the corresponding ketone, which was reduced with $NaBH_4$[22] (to avoid side reactions during the ensuing steps) and deprotected with TFA to give alcohols **E**. Coupling of **E** with dipeptide **F** was effected by DCC, and intermediate **G** was converted to **2** by the methods used for **C** (Scheme 1). Crude **2** from both routes contained 10−20% of the epimeric D-Arg tripeptide,[23] which was removed during reverse-phase HPLC purification. The second route (Scheme 2) is more convergent and more generally applicable. The diastereomeric alcohols corresponding to **2**, **4a**,**b**, were prepared by the HF deprotection of **G** and then separated by HPLC.

Biological Testing. The compounds were tested for inhibition of human α-thrombin and bovine trypsin. The

* Address correspondence to this author. Telefax: 215-628-4985. E-mail: maryanoff@prius.jnj.com.
† R. W. Johnson Pharmaceutical Research Institute.
‡ Michigan State University.

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Table 1. Chemical Properties and Biological Data for Peptidoyl Heterocycles[a]

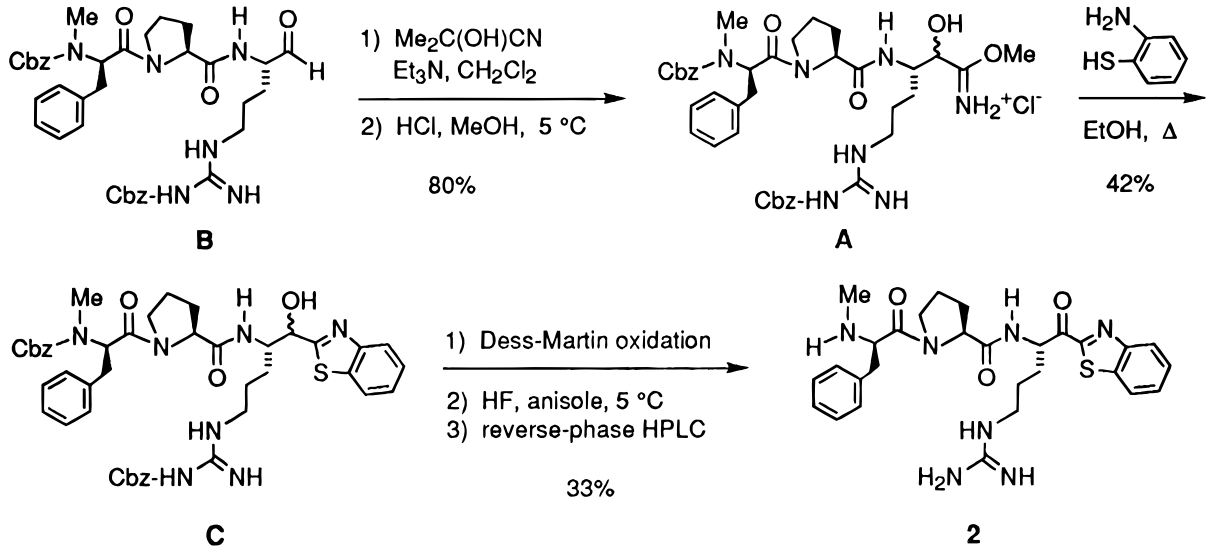
Me-(D-Phe)-Pro-Arg-Het

2, **3a-3i**

compd	Het	mp, °C[b]	$[\alpha]_D$,[c] deg	K_i, nM[d] thr	K_i, nM[d] try	try/thr[e]
2	2-benzothiazole	93−104	−71.2 (*c* 0.76)[f]	0.19 ± 0.07 (7)	3.1 ± 1.7 (3)	16
3a	2-thiazole			3.6 ± 0.3 (3)	1.6 ± 0.7 (2)	0.4
3b	2-benzothiophene		−78.3 (*c* 1.00)	2400 ± 900 (6)	1340 ± 840 (6)	0.6
3c	2-benzoxazole	44−54	−63.3 (*c* 0.58)	3.8 ± 1.4 (6)	2.9 ± 1.9 (5)	0.8
3d	2-H_4-benzothiazole[g]	50−60	−71.7 (*c* 0.69)	3.4 ± 2.1 (6)	4.4 ± 2.4 (5)	1.3
3e	2-benzimidazole	70−80	−63.1 (*c* 1.00)[h]	12.0 ± 1.0 (6)	38 ± 4 (6)	3.2
3f	*N*-Me-2-benzimidazole	70−90	−57.8 (*c* 1.00)[h]	8.1 ± 2.7 (6)	290 ± 90 (6)	36
3g	*N*-Me-2-imidazole	70−83	−63.7 (*c* 1.00)	50 ± 13 (3)	4400 ± 800 (3)	88
3h	2-quinazolin-4-one	122−138		15 ± 6 (6)	8.2 ± 1.4 (6)	0.6
3i[i]	2-pyridine	40−68		85 ± 44 (6)	73 ± 32 (6)	0.8
4a[j]		77−180 dec	−61.8 (*c* 1.21)[f]	5300 ± 2500 (6)	*k*	
4b[j]		65−175 dec	−69.7 (*c* 1.05)[f]	1600 ± 200 (3)	*k*	
efegatran[l]				10.0 ± 5.0 (6)	3.9 ± 1.3 (6)	0.39
argatroban[l]				10.0 ± 2.0 (6)	2900 ± 1200 (6)	290

[a] All new compounds were isolated and purified by reverse-phase HPLC (MeCN:water:TFA, 30:70:0.1) and lyophilized as hydrated trifluoroacetate (TFA) adducts. They are represented by standard molecular formulas with the following solvates: **2** (2.6 TFA, 1.5 water), **3a** (2.0 TFA, 1.2 water), **3b** (2.4 TFA, 1.5 water), **3c** (4.0 TFA, 2.2 water), **3d** (2.6 TFA, 1.4 water), **3e** (3.8 TFA, 1.0 water), **3f** (3.2 TFA, 1.1 water), **3g** (3.9 TFA, 1.1 water), **3h** (2.6 TFA, 3.0 water), **3i** (2.8 TFA, 2.2 water), **4a** (4.0 TFA, 1.5 water), and **4b** (4.2 TFA, 1.8 water). Microanalytical data (C, H, N, and water) were within the accepted range, unless noted otherwise. The new target compounds were characterized by high-field NMR and mass spectrometry. Information on the enzymatic assays is contained in the Supporting Information. [b] Melting point values are corrected. The products were amorphous powders obtained by lyophilization. [c] Optical rotations were determined in MeOH at 25 °C, unless noted otherwise. [d] The standard error is given for *N* measurements, indicated in parentheses. For methodology, see the Supporting Information. [e] Ratio of the K_i value for trypsin to the K_i value for thrombin. [f] Determined at 20 °C. [g] 4,5,6,7-Tetrahydro-2-benzothiazole. [h] Determined in water. [i] Mixture of L- and D-epimers at Arg Cα in a 85:15 ratio. [j] Single diastereomer of unestablished configuration. Water analysis was out of range; calcd/found: 2.61/0.92 for **4a**, 2.95/1.40 for **4b**. [k] Greater than 150 000 nM; $N = 3$. [l] Reference thrombin inhibitor. Efegatran has been called GYKI-14766. Argatroban has been called MD-805 and argipidine.

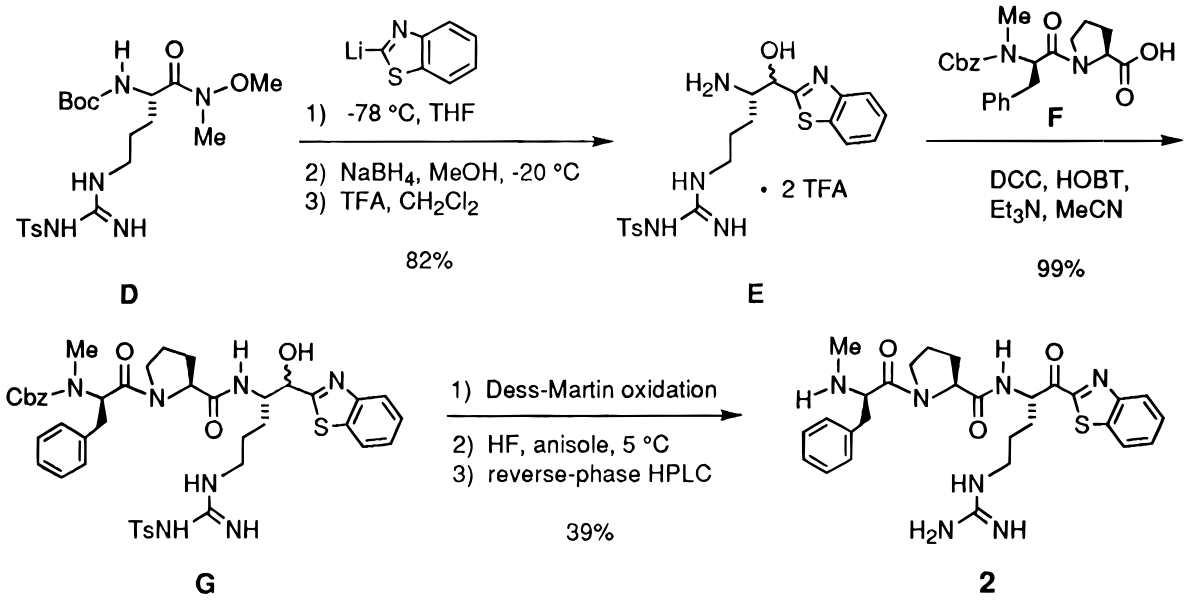
Scheme 1



data in Table 1 illustrate the importance of an aromatic stacking interaction with Trp-60D for high affinity and selectivity, as reflected in the X-ray crystallographic results (*vide infra*): cf. **2** with **3a**,**d** and **3f** with **3g**. Although methyl substitution in the imidazole series did not improve thrombin inhibition, it did increase the selectivity for thrombin over trypsin (entries **3e**−**g**). Two proximal heteroatoms are necessary, but not sufficient, for potent activity, perhaps on account of a requirement for effective activation of the electrophilic ketone (cf. **2** with **3b**,**i**). Although pyridine derivative **3i** has a π-deficient heterocycle that can activate the adjacent carbonyl, it showed fairly modest potency. The significant increase in potency for benzothiazole **2** compared to benzoxazole **3c** (20-fold) and *N*-methylbenzimidazole **3f** (40-fold) may be connected with the relative π electron-withdrawing power of these heterocycles.[24] The results for alcohols **4a**,**b** further support the importance of ketone activation for strong enzyme inhibition, consistent with a transition state analogue.

The excellent thrombin inhibitor **2**, as well as the reference standards efegatran and argatroban, was evaluated for selectivity versus other, important coagulation enzymes: plasmin, tPA, activated protein C, and streptokinase (Table 2). Although **2** is not as selective as argatroban with these four enzymes,[25] it still shows noteworthy selectivity for potential development, and **2** is considerably more selective than efegatran.[11a,26] In the case of trypsin, **2** is a fairly potent inhibitor, affording a selectivity ratio of just 16 in favor of thrombin inhibition. It should be noted that a lack of selectivity for thrombin over trypsin appears to be a

Scheme 2



D **E** **F** **G** **2**

Table 2. Inhibition of Thrombin Relative to Other Serine Proteases[a]

		selectivity (K_i other/K_i thr)[b]				
compd	K_i, nM, thrombin	trypsin	plasmin	tPA	prot Ca	SK
2	0.19 ± 0.07 (7)	16	12 000	3300	19 000	6300
efegatran	10.0 ± 5.0 (6)	0.39	33	130	72	170
argatroban	10.0 ± 2.0 (6)	290	40 000	37 000	>50 000	>50 000

[a] K_i values (nM, mean ± standard error) are given for thrombin inhibition; the number of experiments (N) is in parentheses. [b] Selectivity is defined as the ratio of the K_i value of the serine protease over the K_i value for thrombin. For the original K_i data, see the Supporting Information. tPA, tissue-type plasminogen activator; prot Ca, activated protein C; SK, streptokinase.

general property of inhibitors with a D-Phe-Pro-Arg motif;[7c] nevertheless, the selectivity exhibited by **2** for thrombin over trypsin is significantly better than that by efegatran (try/thr = 0.39), which is currently involved in clinical trials.

We also examined **2** for functional activity with gel-filtered human platelets, in comparison with efegatran and argatroban. Compound **2** inhibited thrombin-induced platelet aggregation with an IC_{50} value of 23 ± 2 nM (N = 5), which compares favorably with the values obtained for efegatran (23 ± 12 nM; N = 3) and argatroban (52 ± 9 nM; N = 3). Also, **2** exhibited potent antithrombotic activity in several animal models on intravenous administration, but the results of this work will be reported separately.

X-ray Crystallographic Structure.[27] A ternary complex was prepared by adding **2** in 10-fold excess to the hirugen−thrombin complex, and crystals were grown by the hanging-drop method.[28] Diffraction data were collected by using a Siemens multiwire X-1000 detector (R_{merge} = 0.055). The monoclinic crystals are isomorphous with crystals of other hirugen−thrombin constructs[28,29] and diffracted X-rays to about 2.3 Å. After ca. 15 cycles of PROLSQ refinement (R-factor = 0.20), the electron density was compatible with two possible orientations of the benzothiazole ring, with interchanged nitrogen and sulfur atoms. The correct position was established by occupancy refinement of the sulfur atom in each position, also revealing a hydrogen bond between N1 of the benzothiazole and NE2 of His-57 of thrombin. Final refinement converged at R = 0.168 with 125 water molecules. Structural features of the active site of **2**−thrombin−hirugen are depicted in Figure 1.

Compound **2** is structurally analogous to PPACK (D-Phe-Pro-Arg-CH_2Cl), but it has a methyl group added to the N-terminus and a 2-benzothiazole in place of the chloromethyl group. The overall interactions for **2**−thrombin are similar to those for PPACK−thrombin (Figure 2).[16] The inhibitor forms a hemiketal adduct between the Ser-195 hydroxyl and the arginine carbonyl, and the tripeptide motif (P_3−P_2−P_1) has the standard interactions previously observed in thrombin−inhibitor complexes of this type. However, the benzothiazole group binds in an interesting manner at the S_1' subsite, which is mainly defined by His-57, Tyr-60A, Trp-60D, and Lys-60F. The benzothiazole ring (P_1') hydrogen bonds via its nitrogen atom to His-57 (N−N distance of 2.7 Å) and stacks in a face-to-edge manner with the indole ring of Trp-60D. Edwards et al.[12] inferred (as the benzoxazole O and N positions could not be crystallographically distinguished) a similar hydrogen bond in the crystal structure of their PPE complex. Remarkably, despite the potent inhibition of thrombin by **2**, the benzothiazole ring is found to displace the side chain of Lys-60F from its normal position in other inhibitor−thrombin complexes, such that the aminobutyl group has folded into a U-shaped, unextended conformation.

Conclusion. We have studied a series of peptidoyl heterocycles with the structure Me-(D-Phe)-Pro-Arg-Het and identified some potent inhibitors of human α-thrombin. The best thrombin inhibitor, **2** (RWJ-50353), possesses a 2-benzothiazole group and has a K_i value of 0.19 nM. This agent has excellent selectivity for

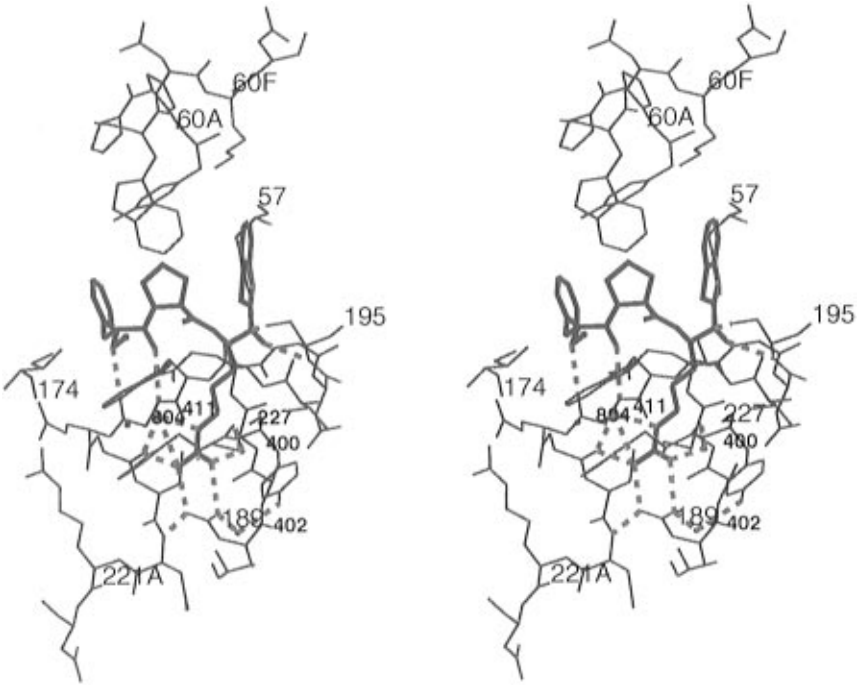


Figure 1. Stereoview of **2** bound in the active-site cleft of thrombin. Color code: blue = thrombin, red = ligand **2**. (The ligand is also shown in bolder typeface.) Hydrogen bonds are indicated by broken red lines. Residues in thrombin are numbered with the larger font, and water molecules are numbered with the smaller font.

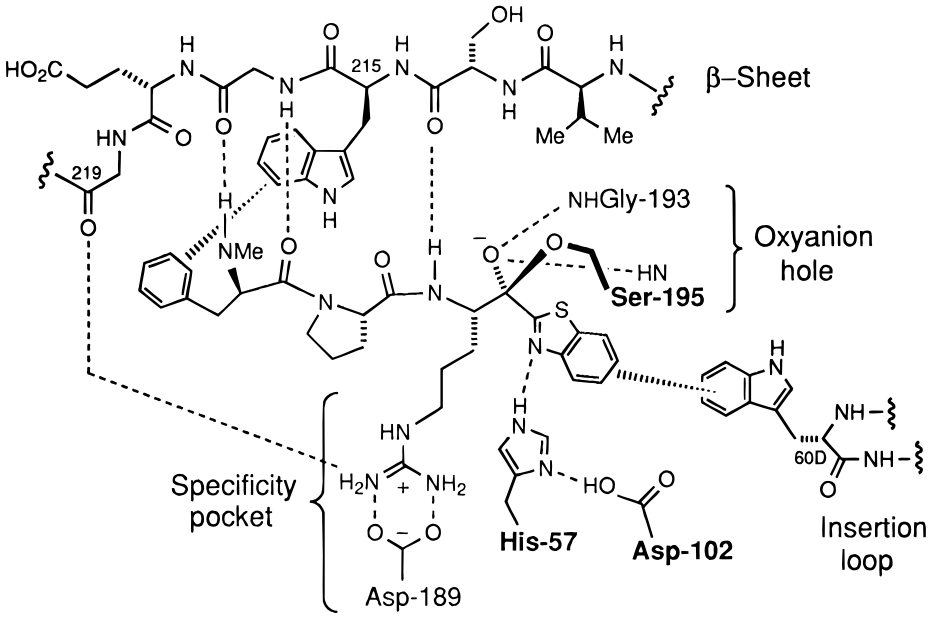


Figure 2. Diagram representing the key interactions between **2** and thrombin.

thrombin vs four other enzymes important to blood coagulation and is a powerful inhibitor of platelet aggregation. The crystallographic structure of a complex between **2** and thrombin reveals novel interactions in the S_1' region, where the benzothiazole forms a hydrogen bond with His-57 and an aromatic stacking interaction with Trp-60D of the insertion loop of thrombin. Subnanomolar inhibition occurs with **2** despite the Lys-60F side chain moving from its normal position to a folded conformation because of the bulky benzothiazole group. Our work extends to thrombin inhibitors the heterocycle-activated carbonyl approach pioneered by Edwards and co-workers for elastase inhibitors[12,13] and further supports the proposed mechanism of binding.

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Supporting Information Available: Details for the enzyme assays and K_i values for Table 2 (2 pages). See any current masthead page for ordering information.

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(1) (a) Presented in part at the 210th National Meeting of the American Chemical Society, Chicago, IL, Aug. 20−24, 1994; ORGN-25. (b) Abbreviations: Cbz, carbobenzoxy; Fmoc, 9-fluorenylmethylcarbonyl; TFA, trifluoroacetic acid; DCC, dicyclohexylcarbodiimide.

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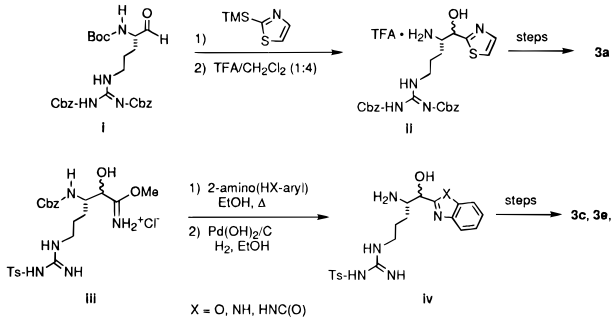
(17) (a) We prepared **3b**,**d**,**f**,**g**,**i** from the appropriate lithio heterocycles by using the methods outlined for **2** in Scheme 2. (b) Preparation of **3a**: Aldehyde **i** was reacted with 2-(trimethylsilyl)thiazole[18] to give **ii**, which was converted to **3a** by the method used for **E** in Scheme 2. (c) Preparation of **3c**,**e**,**h**: Imidate **iii**[11b] was reacted with 2-aminophenol, *o*-phenylenediamine, or anthranilic acid, respectively, followed by hydrogenolysis to afford **iv**, which was converted to targets by the method used for **E** in Scheme 2.



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